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ORIGINAL ARTICLE

The floral volatile, methyl benzoate, from snapdragon (*Antirrhinum majus*) triggers phytotoxic effects in *Arabidopsis thaliana*

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Abstract Previously it has been shown that the floral scent of snapdragon flowers consists of a relatively simple mixture of volatile organic compounds (VOCs). These compounds are thought to be involved in the attraction of pollinators; however, little is known about their effect on other organisms, such as neighboring plants. Here, we report that VOCs from snapdragon flowers inhibit *Arabidopsis* root growth. Out of the three major snapdragon floral volatiles, myrcene, (*E*)-β-ocimene, and methyl benzoate (MB), MB was found to be primarily responsible for the inhibition of root growth. Ten micromoles MB reduced root length by 72.6%. We employed a microarray approach to identify the MB target genes in *Arabidopsis* that were responsible for the root growth inhibition phenotype in response to MB. These analyses showed that MB treatment affected 1.33% of global gene expression, including cytokinin, auxin and other plant-hormone-related genes, and genes related to seed germination processes in *Arabidopsis*. Accordingly, the root growth of cytokinin (*cre1*) and auxin (*axr1*) response mutants was less affected than that of the wild type by the volatile compound: roots of the treated mutants were reduced by 45.1 and 56.2%, respectively, relative to untreated control mutants.

Keywords Snapdragon · Floral volatiles · Allelopathy · Root growth · Methyl benzoate

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Introduction

Plant chemicals known as volatile organic compounds (VOCs) mediate intra-organismal communication, including interactions between plants and insects, and plants and microbes. In some species, leaf VOCs attract enemies of leaf-feeding insects, thereby acting as an indirect plant defense response against insect herbivory (Pare and Tumlinson 1999; Dicke and Van Loon 2000; Horiuchi et al. 2001). It has been recently reported that the VOC (*E*)-β-caryophyllene, released from the roots of maize in response to feeding by larvae of the beetle *Diabrotica virgifera virgifera*, attracts an

entomopathogenic nematode *Heterorhabditis megidis* (Rasmann et al. 2005). Plant VOCs can also have anti-microbial activity that inhibits the spread of plant pathogens (Cardoza et al. 2003; Huang et al. 2003; Holopainen 2004). Volatiles emitted by some plants are also involved in plant–plant communication. Volatiles such as methyl jasmonate, terpenoids, green leaf volatiles, and methyl salicylate are emitted from damaged or attacked leaves and serve as a signal to induce defenses in neighboring plants, thus facilitating positive plant–plant communication (Arimura et al. 2000a; Dicke and Bruin 2001; Engelberth et al. 2004; Shulaev et al. 1997). Furthermore, isoprene emitted from the foliage of many woody species (Sharkey and Yeh 2001) is thought to increase photosynthesis rates, allowing neighboring plants to tolerate high temperatures by stabilizing thylakoid membranes (Sharkey et al. 2001).

Floral volatiles are well-characterized attractants of pollinators (Henderson 1986; Raguso and Pichersky 1995; Jones et al. 1998; Reinhard et al. 2004), but much less is known about the inter-organismal interactions mediated by these volatiles. Some floral volatiles have recently been reported to have anti-microbial or anti-herbivore activity, serving to protect the reproductive organs and their valuable germ line cells against potential foes (Chen et al. 2003; Friedman et al. 2002; Hammer et al. 2003). However, nothing is known about whether floral volatiles have a negative effect on neighboring plants, or what the mechanism of such an effect might be at the physiological and molecular levels.

To determine whether floral scents or individual floral volatiles have allelopathic activity, we chose snapdragon, *Antirrhinum majus* L., as a model system, since its floral scent was already well characterized (Dudareva et al. 2000, 2003; Kolosova et al. 2001; Negre et al. 2003). Here we report that snapdragon floral volatiles affect the root growth of *Arabidopsis thaliana*, and we show that methyl benzoate (MB) is the primary volatile compound responsible for this allelopathic effect; we further discuss the volatile's effect on plants at the molecular transcription level using microarray analysis. Our results indicate that neighboring plants can sense and in this case negatively respond to the effect of flower volatiles.

Materials and methods

Materials and volatile exposure studies

Garden snapdragon seeds (*Antirrhinum majus*) were purchased from Ball Seed (West Chicago, IL, USA). Snapdragon plants were grown from seed in mixed soil [50% peat moss (Canadian Sphagnum) and 50% perlite (Persolite)] in a climate-controlled greenhouse (24°C). MB (99%) and myrcene (>95%) were purchased from Sigma/Aldrich (St. Louis, MO, USA), and (*E*)-β-ocimene (90%) was purchased from International Flavors Fragrances (New York, NY, USA). *Arabidopsis thaliana* (Col-0) and mutant seeds (auxin response mutant: CS3075, ethylene: CS237 and CS8058, gibberellin: CS63, jasmonic acid: CS8072, brassinosteroid: CS291, cytokinin: SALK048970) were purchased from the Lehle Seeds Co. (Round Rock, TX, USA) and TAIR (Stanford, CA, USA). Before planting, seeds were soaked in water for 3 days at 4°C, sterilized in sodium hypochlorite solution (Sigma), and washed three times with sterile distilled water. Ten seeds of *Arabidopsis* or snapdragon were germinated on solid Murashige-Skoog (MS) medium (Murashige and Skoog 1962) with 1.5% agar in each of two sections of a four-section divided plastic dish (VWR, see Supplemental Fig. 1). Two 3- to 5-day-old snapdragon flowers or four mature leaves were collected, and were placed in the other two sections. To apply pure compound treatments, volatile organic compounds were diluted in methanol and 5 µl of each compound was added to a cotton ball in the other two sections instead of the flowers or leaves. As a control, methanol without pure compounds was added to a cotton ball in the other two sections. The dishes were then sealed with parafilm and transferred to a climate-controlled chamber (20°C, 16/8 h light/dark cycle). After 6 days, roots were straightened to their full length, and root and hypocotyl lengths were measured with a ruler. These experiments were repeated three times.

Root and hypocotyl growth in soil

Arabidopsis seeds (Col-0) were soaked in water for 3 days at 4°C. Then ten seeds were dropped on peat pellets (Jiffy products, Canada). Five peat pellets were kept in a lidded glass container, in combination with four to six snapdragon flowers without leaves in a flask (250 ml) of distilled water (see supplemental Fig. 2). Flowers were changed after 1 week. After 2 weeks of exposure to volatiles, *Arabidopsis* seedlings were gently removed with water from the peat pellet and root and hypocotyl lengths were measured. These experiments were repeated three times.

VOC analysis

Collection of volatiles from detached flowers and leaves was performed in a growth chamber under conditions of 21°C, 50% relative humidity, 150 µmol/m^2/s

light intensity, and a 12-h photoperiod. Four 3- to 5-day-old flowers or eight mature leaves were cut from snapdragon plants and transferred to small glass beakers filled with 10 mL of 5% sucrose in water. Emitted volatiles were collected by a closed-loop stripping method for 24 h (Dudareva et al. 2000; Kolosova et al. 2001). Volatiles were eluted from Porapak Q traps (80/100 mesh size; Alltech Associates) with 200 μL of hexane, toluene (2.88 μg) and naphthalene (3.33 μg) added as internal standards. GC analysis was performed with a FinniganMAT GCQ instrument (Thermoquest, San Jose, CA, USA) (injector temperature 230°C, injector volume 1 μL, and split ratio 50:1) using a DB-1 nonpolar capillary column (30 m × 0.25 mm [i.d.]; film thickness 0.25 μm). Ionization energy was set at 70 eV. Column temperature was held at 50°C for one min and then heated to 240°C at 10°C min^{-1}. The mass spectrometer was scanned from 41 to 400 amu. Ambient volatiles collected at the same times as the samples were used as controls. Components were first identified from a computer database containing several thousand mass spectra and were confirmed by comparing retention times and mass spectra with those of authentic standards (Dudareva et al. 2000; Kolosova et al. 2001).

Arabidopsis oligo microarray analysis

Arabidopsis wild-type seeds were soaked in water for 2 days, surface-sterilized with sodium hypochlorite and plated on MS agar plates. After 36 h of incubation, plated seeds were treated with 10 μmol MB. Seeds were collected after 8 h of exposure to MB for total RNA isolation using a Qiagen RNeasy plant mini kit (Qiagen Inc. CA, USA). Seeds treated with methanol served as the control. The concentration of RNA was quantified using a Nanodrop system (Nanodrop, Wilmington, DE, USA) and the integrity estimated by 28S/18S ratio using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). The microarray analysis was performed at the Yale University microarray lab (Keck Biotechnology Resource Laboratory, CT, USA). For each replicate, 2.5 μg of total RNA was used to prepare cDNA using Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and this cDNA was used for the preparation of Cy5-labelled and Cy3-labelled probes using Genisphere Array 900 Kit and Advalitix ArrayBooster DNA Microarray Incubator. Microarray analysis was performed using the *Arabidopsis* Genome Oligo Set Version 1.0, which contains 26,090 70mers representing 26,090 genes and predicted ORFs obtained from the UniGene Database Build At 4, developed at the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/UniGene). The set also includes 12 unique positive and 12 unique negative controls provided in 16 replicates in a 384-well plate. To assess the reproducibility of microarray analysis, each experiment was repeated two times. Genes showing a signal value <600 in both Cy3 and Cy5 channels of the control treatment were not considered for the analysis. Feature extraction and image analysis softwares were used to locate and delineate every spot in the array and to integrate each spot's intensity, filtering and normalization using the Lowess method. A two-sample *t* test using *P* value cut-off 0.05 was applied to identify genes statistically differentially expressed. Gene clustering analysis was performed using Clustal and Treeview software.

Results

Phytotoxicity bioassays under lab conditions

Approximately 88% of the *Arabidopsis* seeds exposed to the volatiles of snapdragon flowers and leaves germinated after six days. *Arabidopsis* germination was not affected by snapdragon volatiles; percent germination among seeds exposed to snapdragon leaf and flower volatiles was comparable to seeds not exposed to volatiles (Fig. 1a). Six days after germination, *Arabidopsis* seedlings not exposed to the VOCs had an average root length of 14.8 mm and an average hypocotyl length of 2.2 mm. However, seedlings treated with snapdragon flower VOCs had significantly shorter roots (4.8 mm, Bonferroni/Dunn's test: $P < 0.0167$, Fig. 1b, c) and hypocotyls (1.9 mm, Bonferroni/Dunn's test: $P < 0.0167$, Fig. 1b, c). *Arabidopsis* seedling growth was not affected by exposure to snapdragon leaf VOCs (mean root length = 13.5 mm, mean hypocotyl length = 2.3 mm, Fig. 1 b, c). The ability of snapdragon flower volatiles to cause inhibition of root growth was also observed in soil. *Arabidopsis* seeds in peat pots were exposed to flower volatiles for 2 weeks by incubating these plants along with snapdragon flowers in an airtight chamber (Supplementary Fig. 2). Under these conditions, root growth was reduced to 68% of the untreated controls (Bonferroni/Dunn's test: $P < 0.0001$), while hypocotyl growth was not affected (104% compared to untreated control, Fig. 1d). The difference between growth inhibition in soil and agar may be due to the growth pattern of plants in these substrates, as both roots and hypocotyls grew differently under soil conditions (mean root length = 6.3 mm, mean hypocotyl length = 7.3 mm) compared to agar conditions (mean root length = 14.8 mm, mean hypocotyl length = 2.2 mm).

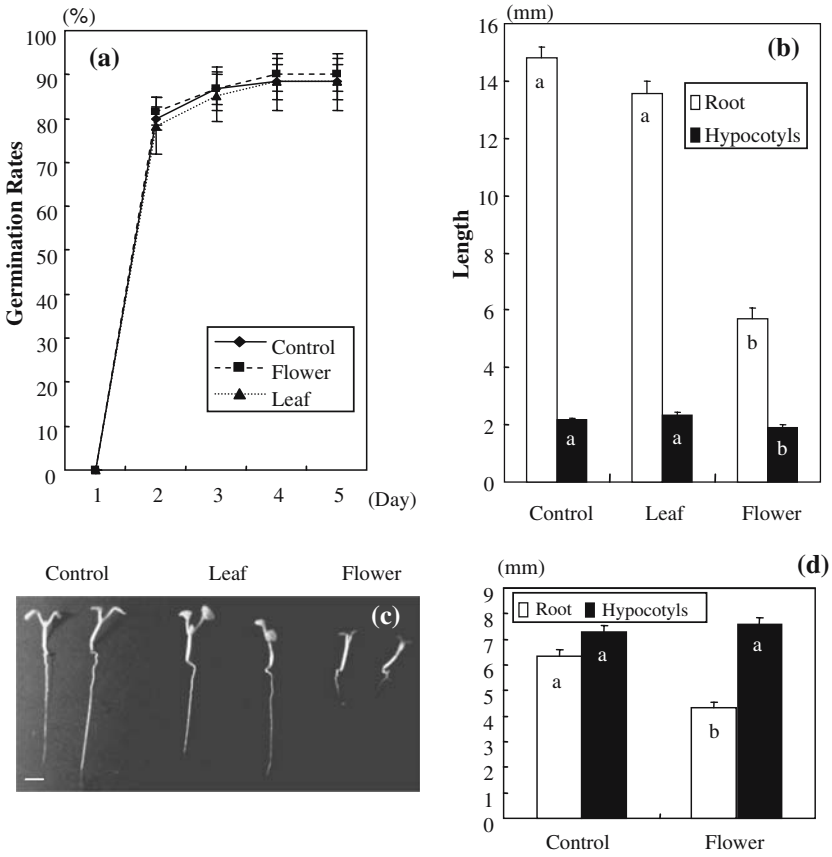

Fig. 1 Seeds of *Arabidopsis* (Col-0) were exposed to snapdragon leaves or flowers. **a** Germination rates ($n = 60$), **b** growth of roots and hypocotyls ($n > 45$), and **c** picture of seedlings after 6 days of treatment. The *white bar* in (**c**) indicates 1 mm. **d** Root and hypocotyl growth after 2 weeks of treatment in soil ($n > 36$). Photos and measurements are representative of three separate experiments. Error bars in (**a**), (**b**) and (**d**) represent one standard error. *Different letters* indicate significantly different mean lengths (Bonferroni/Dunn's test: **b**, 0.0167 level; **d**, 0.0001 level) among bars of the same shading

Effect of snapdragon VOC components on *Arabidopsis* seed germination and root growth

We analyzed the chemical constituents of snapdragon floral volatiles using gas chromatography—mass spectroscopy (GC-MS) analysis. The major components of the flower volatiles were myrcene (about 10%), (*E*)-β-ocimene (>30%), and MB (>35%) (Fig. 2a), consistent with a previous report by Dudareva et al. (2003), while the collection of volatiles from leaves revealed emission of only one compound, β-caryophyllene (Fig. 2b). To determine which component of the snapdragon flower volatiles was involved in root growth inhibition in *Arabidopsis*, we exposed in vitro-grown *Arabidopsis* plants to different concentrations of the individual volatile compounds. Myrcene and (*E*)-β-ocimene had no effect on *Arabidopsis* root and hypocotyl growth (Fig. 3a). However, exposure to MB significantly inhibited root and hypocotyl lengths (Bonferroni/Dunn's test: $P < 0.0083$) in a concentration-dependent manner (Fig. 3a). Root growth of 1 and 10 µmol MB treated seedlings were 73% and 21% of root growth of the control seedlings, respectively (Fig. 3a). With 10 µmol MB, hypocotyl length was reduced to 31% of that of untreated controls (Fig. 3a). Further, MB at 10 µmol was phytotoxic as evidenced by reduced seed germination rates (23%) 6 days after treatment compared to the untreated control's germination rate of 92%, while myrcene and (*E*)-β-ocimene had only minor negative effects on the germination rates (Fig. 3b). Higher levels of MB treatment reduced germination, although snapdragon flower treatment did not reduce germination (Fig. 1a). However, these results strongly suggest that MB is phytotoxic. MB also had a negative effect on snapdragon germination and root growth in a concentration-dependent manner; however, the effect was not as profound as with *Arabidopsis*. Ten micromoles of MB reduced the germination rate (not significant, ANOVA test: $P = 0.3223$), root length (significant, ANOVA test: $P < 0.0001$) and hypocotyl length (significant, ANOVA test: $P < 0.0001$) only to 87, 61 and 56% of that of the controls after 6 days of treatment (Fig. 3c, d).

Methyl benzoate alters global gene expression in *Arabidopsis*

To understand the effects of MB at the gene expression level in *Arabidopsis* we performed global gene expression analysis using a microarray representing 26,090 genes on *Arabidopsis* seeds treated with MB.

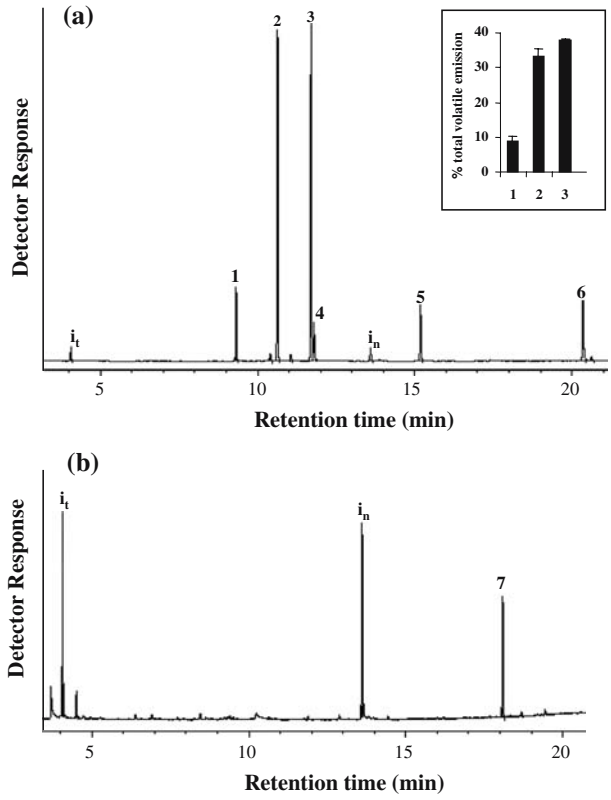

Fig. 2 GC-MS profiles of compounds in the headspace of snapdragon **a** flowers and **b** leaves, and the relative level of emission of each compound in the flower headspace (*inset*): it, toluene (internal standard); in, naphthalene (internal standard); *1* myrcene; *2* E-β-ocimene; *3* methyl benzoate; *4* linalool; *5* 3,5-dimethoxytoluene; *6* nerolidol; *7* caryophyllene

The microarray data analysis suggests that MB treatment significantly ($P < 0.05$) altered the expression of 375 genes (~1.33% of the total genome). 1.1% of the genome of *Arabidopsis* was induced while 0.23% of the genes were repressed by MB (Fig. 4a). Our microarray analysis showed that genes related to hormones such as auxin (At3g44300, At2g33310, At3g60690, At4g27450, At3g04730), cytokinin (At3g04280), gibberellic acid [GA] (At5g25900), abscisic acid [ABA] (At3g02480, At2g40170), and ethylene (At1g43160, At5g61600, At4g21340, At5g43450, At3g20770, At5g61590) were significantly up-regulated by MB treatment (Fig. 4b and Supplemental Table 1). In addition to these hormone-related genes, we found other functional protein families up-regulated in expression patterns by MB, including the WD-40 protein family, the Ubiquitin protein family, the Late Embryogenesis Abundant (LEA) protein family, the No Apical Meristem (NAM) protein family, ABC transporters, cysteine proteases, Myb transcription factors and other transcription factors.

Response of *Arabidopsis* hormone-deficient mutants to methyl benzoate

Based on the previous micro-array data and to probe the mechanism by which MB can induce a phytotoxic effect on plants, we applied 10 μmol MB to several *Arabidopsis* hormone response mutants. Jasmonic acid (*jar1*), gibberellin (*gai1*) and ethylene (*etr1*) response mutants showed similar germination inhibition to wild-type plants. After 6 days, mutant seed germination rates in the presence of MB were 33.3, 42.1 and 31.6%, for *jar1*, *gai1*, and *etr1* respectively, of germination rates of the mutant seeds without MB (Fig. 5a). Germination of brassinosteroid (*cbb1*) and ethylene (*eir1* and *ein2*) response mutants after 6 days of MB treatment were also affected by MB exposure, but to a lesser extent: germination rates of the treated plants were 68.5, 70.0 and 82.1%, respectively, of germination rates of the mutant seeds without MB (Fig. 5a). However, germination of cytokinin (*cre1*), auxin (*axr1*), and another ethylene (*ein2*) response mutants were hardly affected by MB treatment; germination rates of the mutants after 6 days of MB treatment were 94, 102 and 82%, respectively, of the germination rates of the mutant seeds without MB. There were no significant differences between the germination rates of any of the mutants treated for 6 days with MB or without MB (Fig. 5a).

The root lengths of jasmonic acid (*jar1*), gibberellin (*gai1*) and ethylene (*etr1*) response mutants exposed to MB were 2.6, 2.0 and 4.8 mm, respectively, and these root lengths were 17.2, 10.1 and 30.3% of the root lengths of the mutants without MB. The root lengths of the brassinosteroid (*cbb1*) and ethylene (*eir1* and *ein2*) response mutants exposed to MB were 2.2, 4.1 and 5.4 mm, respectively: these root lengths were 32.4, 27.9 and 30.2% of those of the control mutant without MB. The lowest inhibition was found in the cytokinin (*cre1*) and auxin (*axr1*) response mutants; the root lengths of the MB-treated mutants were 11.3 and 8.1 mm, respectively: the root lengths of the VOC-treated seeds were 54.9 and 43.8% of the root lengths of control mutants without MB, although the cytokinin response mutant with MB had significantly different root length compared to the wild type (ANOVA test: $P < 0.0001$) and the auxin response mutant did not (ANOVA test: $P = 0.196$).

Discussion

The present study investigates whether the volatiles emitted from snapdragon flowers could have a negative effect on neighboring plants, and as a first step towards validating this hypothesis we used the model plant

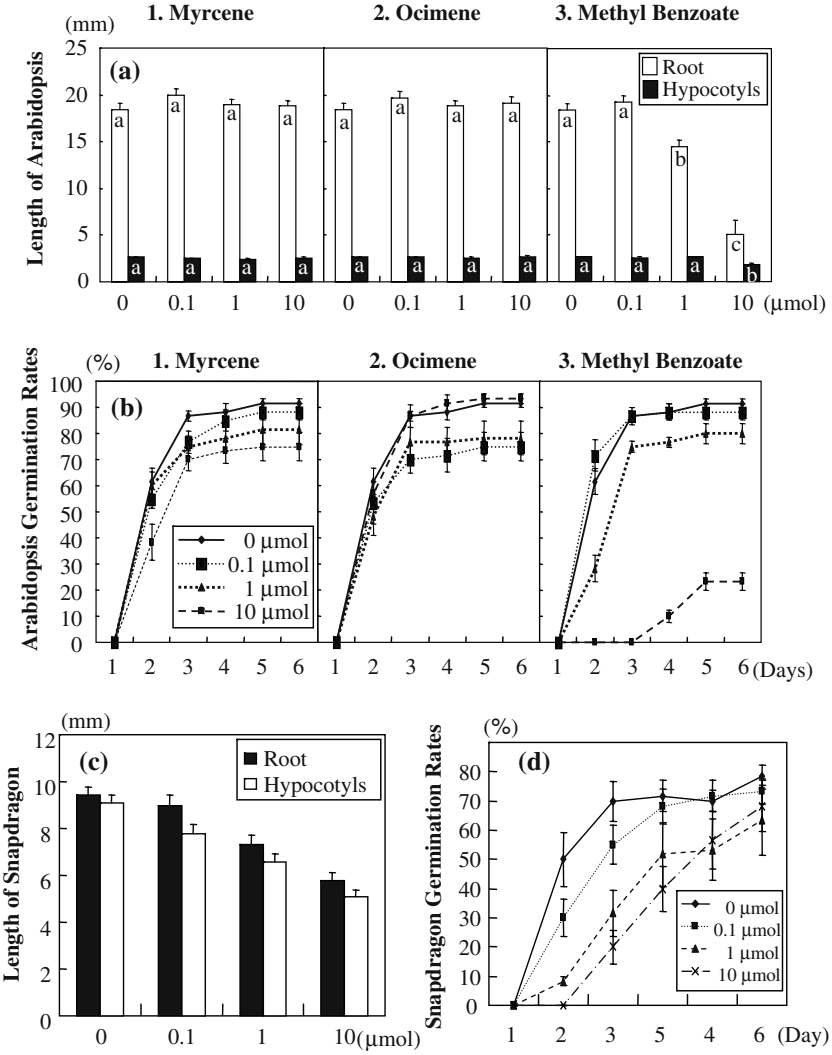

Fig. 3 **a** Root and hypocotyl lengths, and **b** germination rates of *Arabidopsis* (Col-0) in the presence of myrcene, ocimene, and methyl benzoate. **c** Root and hypocotyl lengths, and **d** germination rates of snapdragon in the presence of methyl benzoate. Root and hypocotyl lengths were measured after 6 days of treatment with the volatiles. Zero-micromole means methanol treatment without volatile compounds. *Each bar* represents the mean of measurements of over 38 plants. Error bars are one standard error. In panel (**a**), *different letters* indicate significantly different means (Bonferroni/Dunn's test: 0.0083 level) among bars of the same shading

Arabidopsis as a target. Our results show that exposure of *Arabidopsis* to snapdragon floral volatiles inhibits seedlings' root growth. We found that out of the three major volatile constituents of snapdragon floral scent, MB alone is sufficient to cause root growth inhibition. Although it is not known how closely our MB application corresponds to the real conditions in the field, the chosen concentration of MB for treatments was in the range of the amount emitted from four flowers over a 24-h period (1.3 μmol, Dudareva et al. 2000; Kolosova et al. 2001).

Plant volatiles are believed to have allelopathic (phytotoxic) effects on other plants. Field studies have indicated that the volatile 1,8-cineole released by *Salvia leucophylla* inhibits the growth of neighboring plants (Muller et al. 1964), although other factors also contribute to this effect (Bartholomew 1970). In laboratory experiments, 1,8-cineole has been identified as one of the most potent allelochemicals released by *Artemisia* spp. (Halligan 1975) and *Eucalyptus* spp. (Kumar and Motto 1986). Volatiles released from leaves of *Artemisia tridentata* Nutt. var. *vaseyana* (Weaver and Klovich 1977) and *Sasa cernua* Makino (Li et al. 1992) inhibited the growth of barley seedlings and decreased the respiration rate of germinating seeds. Furthermore, a number of studies have indicated that plant VOCs, such as methyl jasmonate and trans-2-hexenal, are capable of inhibiting plant growth (Staswick et al. 1992; Bate and Rothstein 1998). Here, we present evidence that the floral scent of garden snapdragon inhibits root growth of *Arabidopsis*, while having little effect on snapdragon growth. Interestingly, two other members of the Scrophulariaceae family, yellow toadflax (*Linaria vulgaris*) and Dalmatian toadflax (*Linaria genistifolia* spp. *dalmatica*), are known to be aggressive invasive weeds in North America, and may present valid models for testing the biological effects reported in this study under realistic field conditions, if they emit MB or other allelopathic VOCs.

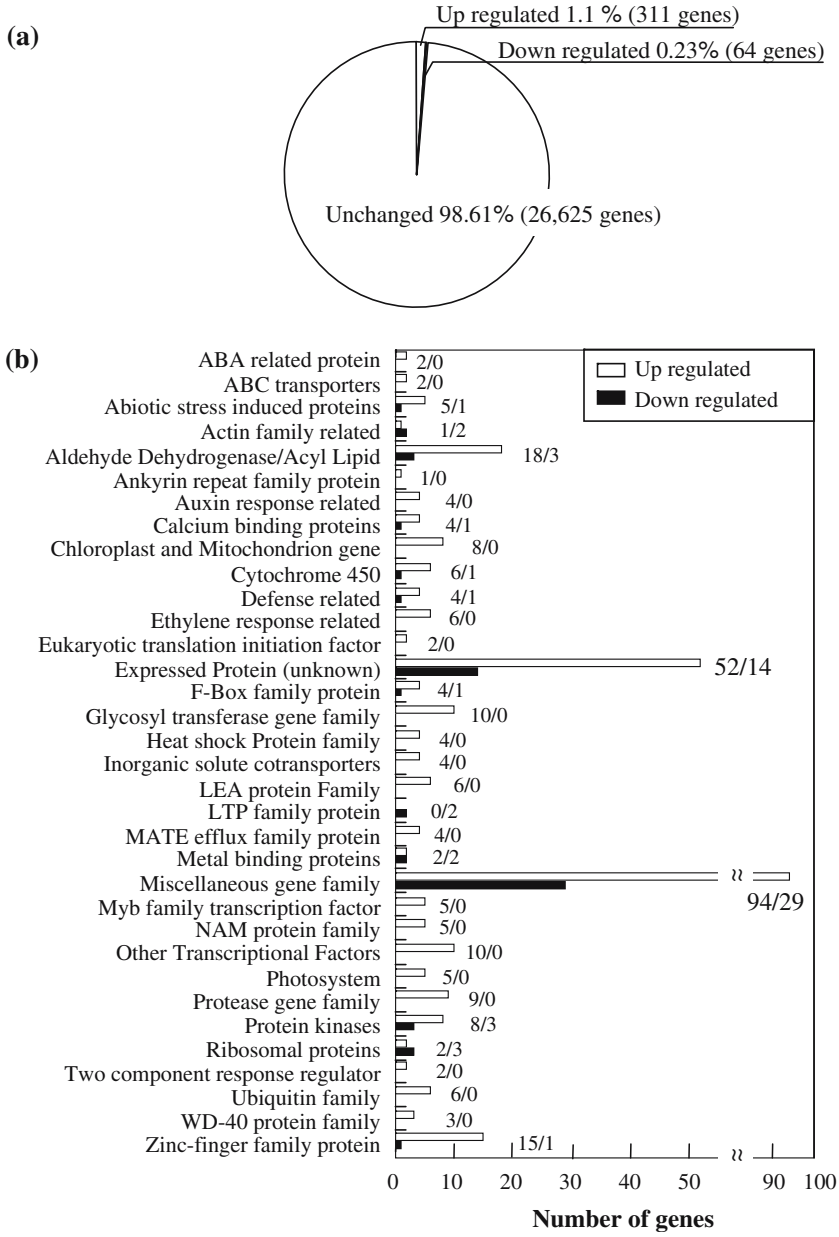

Fig. 4 Microarray analysis of *Arabidopsis* seeds treated with VOC MB. **a** Total number of genes either affected (up-regulated: increased twofold more than control, down-regulated: decreased twofold more than control) or unaffected by treatment with MB, which significantly ($P < 0.05$) affected the expression of 375 genes. **b** Functional categories of genes that are either induced or repressed in *Arabidopsis* upon MB treatment

Methyl benzoate, the volatile identified as primarily responsible for the allelopathic effects observed, is synthesized by enzymatic methylation of benzoic acid in a reaction catalyzed by *S*-adenosyl-L-methionine:benzoic acid carboxyl methyltransferase (Dudareva et al. 2000; Murfitt et al. 2000). This volatile ester is emitted mainly from the upper and lower lobes of petals where pollinators come into contact with the flower (Dudareva et al. 2000) and is thought to be one of the attractants of pollinators (Jones et al. 1998). Furthermore, it is reported that MB inhibits mycelial growth and aflatoxin release by fungi (Chipley and Uraih 1980), and is cytotoxic to bluegill sunfish BF-2 cells (Shen et al. 1998). In our experiments MB inhibited both germination and root growth in *Arabidopsis*. To investigate the molecular aspects of the *Arabidopsis* response to MB treatment, we used a microarray approach to dissect the possible functional mechanisms involved. To our knowledge this is the first report that examines the effects of a single volatile compound on plants using microarray analysis, although the effect of the volatile mixture released from lima bean plants on other lima bean plants has been examined using microarray analysis (Arimura et al. 2000b). Our microarray data analysis showed that certain gene families were up-regulated following MB treatment, particularly the gene families related to the biosynthesis, signaling, and response of hormones such as auxin, gibberellin, cytokinin and

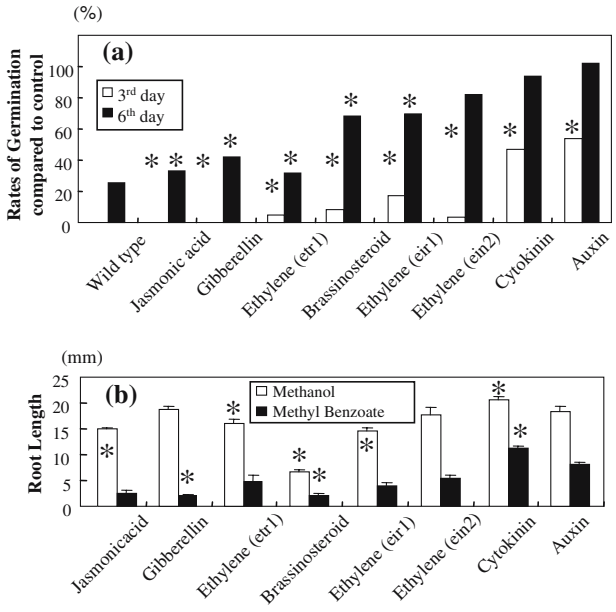

Fig. 5 **a** Rates of germination compared to mutant without MB ($n > 40$) and **b** root lengths ($n > 12$) of *Arabidopsis* hormone-response mutants in the presence of 10 mmol methyl benzoate. Root lengths were measured 6 days after treatment with the volatile. Error bars indicate one standard error. In panel (**a**), the *asterisks* indicate a significant difference between mutant germination rates with MB and without MB (ANOVA test: $P < 0.05$). In panel (**b**), the *asterisks* indicate a significant difference between mutant root length and wild-type root length with the same treatment (ANOVA test: $P < 0.05$). Auxin: *axr1*, Cytokinin: *cre1*, Gibberellin: *gai1*, Brasinosteroid: *cbb1*, Jasmonic acid: *jar1*, Ethylene (*etr1*): *etr1*, Ethylene (ein2): *ein2*, Ethylene (eir1): *eir1*

ethylene. These are essential phytohormones that control various aspects of plant growth and development including root growth (Grabov et al. 2004; Mallory et al. 2005; Nishimura et al. 2004). Moreover, studies have demonstrated the existence of synergistic, antagonistic, and additive interactions between these plant hormones (Coenen and Lomax 1997). Our results show that in wild-type *Arabidopsis* three auxin-response-related genes (two IAA inducible and one calcium inducible) and one cytokinin-response-related gene (Hwang and Sheen 2001; Hwang et al. 2002) were induced by MB treatment. These results are in agreement with those obtained by our hormone response mutant germination and root growth trials in the presence and absence of MB (Fig. 5).

Previous studies have shown that seed germination is determined by the net effect of multiple hormones at critical levels and suggest the importance of interactions between GA, ABA and ethylene (Karssen et al. 1989; Kepczynski and Kepczynska 1997). Leung and Giraudat (1998) reported that ABA plays a critical role in the maintenance of seed dormancy and inhibits germination. Our microarray analysis showed that auxin-, GA-, ABA-, and ethylene-related genes were up-regulated by MB treatment (Fig. 4b and Supplemental Table 1, GA: 1 At5g25900, ABA: 2, Ethylene: 6). Apart from these up-regulated genes related to hormones, we found that other functional protein families related to seed germination, including WD-40 protein family, the ubiquitin protein family, the LEA protein family, and Myb transcription factors were up-regulated upon MB treatment. These protein families are known to be involved in various aspects of seed imbibition and germination processes (Gallardo et al. 2001). Our results are in agreement with the report of Gallardo et al. (2001). For example, LEA proteins are accumulated late during embryogenesis and coincide with the acquisition of desiccation tolerance of developing seeds during germination. They are presumably involved in the binding or replacement of water and in sequestering ions that accumulate during dehydration, and they appear to be developmentally regulated, being abundant in dry mature seeds and disappearing during germination (Wehmeyer et al. 1996).

It has been shown previously that some plant VOCs have an effect on the growth and seed germination of other plants (Muller and Muller 1964; Asplund 1968; Duke and Abbas 1995). Bate and Rothstein (1998) showed that aerial *trans*-2-hexenal quantitatively reduced *Arabidopsis* seed germination and root growth, similar to methyl jasmonate. *Trans*-2-hexenal also reduced the germination frequency and root growth of the methyl-jasmonate-resistant *Arabidopsis* mutant, *jar1* (Staswick et al. 2002). Thus, it was suggested that *trans*-2-hexenal and methyl jasmonate are recognized via different mechanisms (Bate and Rothstein 1998). In our experiment, *jar1* mutants treated with MB did not show recovered germination rates or root growth (Fig. 5), suggesting that the phyto-inhibitory signaling initiated by MB response is not related to the methyl jasmonate pathway. Auxin and cytokinin response mutants had similar rates of germination after 6 days with and without MB, and MB reduced the root growth of these hormone-response mutants to a much lesser extent than the other mutants tested (Fig. 5). The relatively minor effects of MB on auxin and cytokinin response mutant seedlings compared to the other mutants suggests that root growth and germination inhibition in response to MB treatment is regulated via auxin and cytokinin signaling pathways. Although auxin and cytokinin response mutants showed the least response to MB treatment, germination of the brassionosteroid (*cbb1*) response mutants and the ethylene (*ein2* and *eir1*) response mutants other than *etr1* were also less inhibited by MB treatment than the wild type,

suggesting that brassionosteroid and ethylene signaling pathways downstream of *etr1* may also be involved in MB-related germination inhibition (Fig. 5a). Interestingly, the *ein2* and *eir1* mutants were reported to be insensitive not only to ethylene but also cytokinin and auxin (Cary et al. 1995; Luschnig et al. 1998), supporting our conclusion that plant responses to MB occur via cytokinin and auxin signaling pathways. Ryu et al. 2003 reported that plant growth-promoting rhizobacteria release 2,3-butanediol, and that this compound triggers *Arabidopsis* growth promotion via cytokinin-signaling pathways. It appears possible that plants are able to sense and differentially respond to VOCs by turning on different signaling pathways.

Taken together, our results show that MB, a volatile compound emitted from the snapdragon flower, inhibits *Arabidopsis* root growth, indicating that floral volatiles have allelopathic activity.

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References

Arimura G, Ozawa R, Shimoda T, Nishioka T, Boland W, Takabayashi J (2000a) Herbivory-induced volatiles elicit defence genes in lima bean leaves. Nature 6795:512–515

Arimura G, Tashiro K, Kuhara S, Nishioka T, Ozawa R, Takabayashi J (2000b) Gene response in bean leaves induced by herbivory and by herbivory-induced volatiles. Biochem Biophys Res Commun 277:305–310

Asplund RO (1968) Monoterpenes: relationship between structure and inhibition of germination. Phytochemistry 7:1995–1997

Bate NJ, Rothstein SJ (1998) C6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. Plant J 16:561–569

Bartholomew B (1970) Bare zone between California shrub and grassland communities: The role of animals. Science 170:1210–1212

Cardoza YJ, Teal PEA, Tumlinson JH (2003) Effect of peanut plant fungal infection on oviposition preference by *Spodoptera exigua* and on host-searching behavior by *Cotesia marginiventris.* Environ Entmol 32:970–976

Cary AJ, Liu W, Howell SH (1995) Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in *Arabidopsis thaliana* seedlings. Plant Physiol 107:1075–1082

Chen F, Tholl D, D'Auria JC, Farooq A, Pichersky E, Gershenzon J (2003) Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers. Plant Cell 15:481–494

Chipley JR, Uraih N (1980) Inhibition of Aspergillus growth and aflatoxin release by derivatives of benzoic acid. Appl Environ Microbiol 40:352–357

Coenen C, Lomax TL (1997) Auxin—cytokinin interactions in higher plants: old problems and new tools. Trends Plant Sci 2:351–356

Dicke M, Bruin J (2001) Chemical information transfer between plants: back to the future. Biochem Syst Ecol 29:981–994

Dicke M, Van Loon JJA (2000) Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. Entmol Exp Appl 97:237–249

Dudareva N, Murfitt LM, Mann CJ, Gorenstein N, Kolosova N, Kish CM, Bonham C, Wood K (2000) Developmental regulation of methyl benzoate biosynthesis and emission in snapdragon flowers. Plant Cell 12:949–961

Dudareva N, Martin D, Kish CM, Kolosova N, Gorenstein N, Faldt J, Miller B, Bohlmann J (2003) (E)-beta-ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: function and expression of three terpene synthase genes of a new terpene synthase subfamily. Plant Cell 15:1227–1241

Duke SO, Abbas HK (1995) Natural products with potential use as herbicides. Am Chem Soc Symp Ser 582:348–362

Engelberth J, Alborn HT, Swchmelz EA, Tumlinson JH (2004) Airborne signals prime plants against insect herbivore attack. Proc Natl Acad Sci USA 101:1781–1785

Friedman M, Heika PR, Mandrell RE (2002) Bacterial activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. J Food Prot 65:1545–1560

Gallardo K, Joc C, Groot SPC, Puype M, Demol H, Vandekerckhove J, Job D (2001) Proteomic analysis of *Arabidopsis* seed germination and Priming. Plant Physiol 126:835–848

Grabov A, Ashley MK, Rigas S, Hatzopoulos P, Dolan L, Vicente-Agullo F (2004) Morphometric analysis of root shape. New Phytologist 165:641–652

Halligan JP (1975) Toxic terpenes from *Artemisia californica*. Ecology 56:999–1003

Hammer KA, Carson CF, Riley TV (2003) Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil. J Appl Microbiol 95:835–860

Henderson A (1986) A review of pollination studies in the Palmae. Bot Rev 52:221–259

Holopainen JK (2004) Multiple functions of inducible plant volatiles. Trends Plant Sci 9:529–533

Horiuchi J, Arimura G, Ozawa R, Shimoda T, Takabayashi J, Nishioka T.(2001) Exogenous ACC enhances volatiles production mediated by jasmonic acid in lima bean leaves. FEBS Lett 509:332–336

Huang J, Cardoza YJ, Schmelz EA, Raina R, Engelberth J, Tumlinson JH (2003) Differential volatile emissions and salicylic acid levels from tobacco plants in response to different strains of *Pseudomonas syringae*. Planta 217:767–775

Hwang I, Sheen J (2001) Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature 413:383–389

Hwang I, Chen HC, Sheen J (2002) Two-component signal transduction pathways in *Arabidopsis*. Plant Physiol 129:500–515

Jones KN, Reithel JS, Irwin RE (1998) A trade-off between the frequency and duration of bumblebee visits to flowers. Oecologia 117:161–168

Karssen C, Zagorski S, Kepczynski J, Groot S (1989) Key role of endogenous geibberellins in the control of seed germination. Ann Bot 63:71–80

Kepczynski J, Kepczynska E (1997) Ethylene in seed dormancy and germination. Physiol Plant 101:720–726

Kolosova N, Gorenstein N, Kish CM, Dudareva N (2001) Regulation of circadian methyl benzoate emission in diurnally and nocturnally emitting plants. Plant Cell 13:2333–2347

Kumar N, Motto MG (1986) Volatile constituents of peony flowers. Phytochemistry 3:663–671

Leung J, Giraudat J (1998) Abscisic acid signal transduction. Annu Rev Plant Physiol Plant Mol Bio 49:199–222

Li HH, Nishimura H, Hasegawa K, Mizutani J (1992) Allelopathy of *Sasa cernua*. J Chem Ecol 18:1785–1796

Luschnig C, Gaxiola RA, Grisafi P, Fink GR (1998) EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. Genes Dev 12:2175–2187

Mallory AC, Bartel DP, Bartel B (2005) MicroRNA-directed regulation of *Arabidopsis* AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes. Plant Cell 17:1360–1375

Muller WH, Muller CH (1964) Volatile growth inhibitors produced by Salvia species. Bull Torrey Bot Club 91:327–330

Muller CH, Muller WH, Haines BL (1964) Volatile growth inhibitors produced by shrubs. Science 143:471–473

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tissue culture. Physiol Plant 15:473–497

Murfitt LM, Kolosova N, Mann CJ, Dudareva N (2000) Purification and characterization of *S*-adenosyl-L-methionine:benzoic acid carboxyl methyltransferase, the enzyme responsible for biosynthesis of the volatile ester methyl benzoate in flowers of *Antirrhinum majus*. Arch Biochem Biophys 382:145–151

Negre F, Kish CM, Boatright J, Underwood B, Shibuya K, Wagner C, Clark DG, Dudareva N (2003) Regulation of methylbenzoate emission after pollination in snapdragon and petunia flowers. Plant Cell 15:2992–3006

Nishimura C, Ohashi Y, Sato S, Kato T, Tabata S, Ueguchi C (2004) Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in *Arabidopsis*. Plant Cell 16:1365–1377

Pare PW, Tumlinson JH (1999) Plant volatiles as a defense against insect herbivores. Plant Physiol 121:325–331

Raguso RA, Pichersky E (1995) Floral volatiles from *Clarkia breweri* and *C. concinna* (Onagraceae): recent evolution of floral scent and moth pollination. Plant Syst Evol 194:55–67

Rasmann S, Kollner TG, Degenhardt J, Hiltpold I, Toepfer S, Kuhlmann U, Gershenzon J, Turlings TC (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 434:732–737

Reinhard J, Srivivasan MV, Zhang S (2004) Scent-triggered navigation in honeybees. Nature 427:411

Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Pare PW, Kloepper JW (2003) Bacterial volatiles promote growth in *Arabidopsis*. Proc Natl Acad Sci USA 100:4927–4932

Shulaev V, Silverman P, Raskin I (1997) Airborne signaling by methyl salicylate in plant pathogen resistance. Nature 385:718–721

Sharkey TD, Yeh S (2001) Isoprene emission from plants. Annu Rev Plant Physiol Plant Mol Biol 52:407–436

Sharkey TD, Chen X, Yeh S (2001) Isoprene increases thermotolerance of fosmidomycin-fed leaves. Plant Physiol 125:2001–2006

Shen Y, West C, Hutchins SR (1998) In vitro cytotoxicity of aromatic aerobic biotransformation products in bluegill sunfish BF-2 cells. Ecotoxic Environ Safety 45:27–32

Staswick PE, Su W, Howell SH (1992) Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc Natl Acad Sci USA 89:6837–6840

Staswick PE, Tiryaki I, Rowe ML (2002) Jasmonate response locus JAR1 and several related *Arabidopsis* genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell 14:1405–1415

Weaver TW, Klovich D (1977) Allelopathic effects of volatile substances from *Artemisia tridentata* Nutt. Am Midland Naturalist 97:508–512

Wehmeyer N, Hernandez LD, Finkelstein RR, Vierling E (1996) Synthesis of small heat-shock proteins is part of the developmental program of late seed maturation. Plant Physiol 112:747–757